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Award Number: DAMD17-03-1-0341

TITLE: Mechanism of Hormonal Regulation of CAD Gene Expression in Human Breast Cancer Cells

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College Station, TX 77843-3578

REPORT DATE: May 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-05-2006	Annual Summary	1 May 2003 – 30 Apr 2006

4. TITLE AND SUBTITLE

Mechanism of Hormonal Regulation of CAD Gene Expression in Human Breast Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0341

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Shaheen Khan

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Texas A & M Research Foundation
College Station, TX 77843-3578

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

The CAD gene is trifunctional and expresses carbamoylphosphate synthetase/aspartate carbamyltransferase/dihydroorotase, which are required for pyrimidine biosynthesis. TCDD inhibited hormone-induced activation of CAD mRNA levels and pCAD promoter-reporter gene activity in MCF-7 and ZR-75 cells. Using fluorescence resonance energy transfer (FRET), it was shown that both E2 and TCDD enhanced AhR-ER interactions. E2 also induced interactions between ER and Sp1, however cotreatment with TCDD abrogated this effect. Results of this study demonstrate a unique model of AhR-ER crosstalk where the liganded AhR inhibits ER-Sp1 interactions and also recruits ER to Ah-responsive gene promoters such as CYP1A1. In addition CAD gene was also used as a model to study the mechanism underlying the crosstalk between ER and PPAR signaling pathways. PPAR ligand PGJ2 inhibited E2-induced CAD gene expression and also downregulated E2-mediated transactivation of CAD gene promoter constructs and this was reversed by PPAR antagonist T007 in MCF-7 cells. This suggests a possible inhibitory crosstalk between PPAR and ER signaling pathways in breast cancer cells. In addition, 1,1-Bis(3'indolyl)-1-(p-substitutedphenyl)methanes containing p-t-butyl (DIM-C-pPhtBu), and p-phenyl (DIM-C-pPhC6H5) groups, a new class of PPAR agonists, inhibited E2-mediated transactivation of CAD gene promoter constructs, however this effect was PPAR-independent.

15. SUBJECT TERMS
Breast cancer

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	35	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

Estrogen receptor α (ERα) and ERβ are members of the nuclear receptor superfamily of transcription factors, and studies in ER knockout mice and humans show the important role for this receptor in reproductive tract development, neuronal and vascular function, and bone growth. ER expression and activation by estrogens also plays a pivotal role in mammary tumor development and growth (Clemons and Goss, 2001; Hulka et al., 1994) and early stage ER-positive breast cancer has been successfully treated with antiestrogens such as tamoxifen and other selective ER modulators (Jordan, 2003). Although tamoxifen has been extensively used in clinical applications, there is evidence that prolonged use may lead to an increased risk for endometrial cancer or development of tumors resistant to endocrine therapy (Clarke et al., 2001). An alternative approach for inhibiting estrogen-dependent mammary tumor growth using ligands for the aryl hydrocarbon receptor (AhR) has been investigated in this laboratory (Mcdougal et al., 2001). For example, the AhR agonist 6-methyl-1,3,8- trichlorodibenzofuran (6-MCDF) activates inhibitory AhR-ERα crosstalk in breast and endometrial cancer cells, the rodent uterus, and rodent mammary tumors *in vivo* (Mcdougal et al., 2001) and 6- MCDF significantly inhibited 7,12-dimethylbenz[a]anthracene-induced mammary tumor growth in female Sprague Dawley rats at doses as low as 50 μg/kg/d. Moreover, in combination with tamoxifen, 6-MCDF synergistically inhibited mammary tumor growth in the rat model and protected against tamoxifen-induced estrogenic responses in the uterus but did not affect bone lengthening induced by tamoxifen.

Hormone-mediated mammary tumor growth is dependent on modulation of gene expression and in breast cancer cells, AhR agonists, such as 6-MCDF or the high affinity AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), inhibit 17β-estradiol (E2)-induced progesterone receptor, prolactin receptor, cathepsin D, heat shock protein 27, c-*fos*, pS2 and cyclin D1 mRNA and/or protein expression. Based on results of promoter analysis, one inhibitory mechanism involves direct interaction of the AhR complex with inhibitory dioxin responsive elements (iDREs) in E2-responsive gene promoters (Porter et al., 2001). Both E2 and TCDD induce proteasome-dependent degradation of ERα (Wormke et al., 2003) and in breast cancer cells cotreated with E2 plus TCDD, the resulting low levels of ERα may become limiting and thereby decrease expression of some hormone-dependent genes. The first objective of this study was to investigate the mechanism of inhibitory AhR-ERα crosstalk using the hormone-responsive tri-functional carbamoylphosphate synthetase/aspartate carbamyltransferase/dihydroorotase (*CAD*) gene as a model (Khan et al., 2003). Hormonal activation of *CAD* and a number of other E2-responsive genes involved in nucleotide biosynthesis and cell cycle progression is dependent on ERα/Sp1 interactions with GC-rich promoter sequences (Abdelrahim et al., 2002). In this study, we show that TCDD inhibits hormone induced activation of *CAD* mRNA levels and reporter gene activity in MCF-7 and ZR-75 breast cancer cells transfected with constructs (pCAD) containing E2-responsive regions of the proximal region of the *CAD* gene promoter. E2-mediated transactivation of pCAD constructs with a mutant inhibitory dioxin responsive element DRE (iDRE) were also inhibited by TCDD suggesting that inhibitory AhR-ERα/Sp1 crosstalk was iDRE-independent. It was not possible to determine whether the levels of ERα in cells cotreated with E2 plus TCDD

were limiting since the proteasome inhibitor such as MG132 itself directly decreased *CAD* mRNA levels. Using fluorescence resonance energy transfer (FRET), it was shown that both E2 and TCDD enhanced AhR-ERα interactions. E2 also induced interactions between ERα and Sp1; however, cotreatment with TCDD abrogated this effect. Results of chromatin immunoprecipitation assays of the *CAD* gene promoter coupled with the transactivation and FRET data demonstrate a unique model of AhR-ERα crosstalk where the liganded AhR inhibits ERα-Sp1 interactions and also recruits ERα to Ah-responsive gene promoters (e.g. *CYP1A1*).

The second objective of this study was to investigate the mechanisms underlying the inhibitory ER and peroxisome proliferator-activated receptor gamma (PPARγ) crosstalk using *CAD* gene as the model. PPARγ is widely expressed in multiple tumors and cell lines, and this receptor has also become a target for developing new anticancer drugs that will take advantage of the antiproliferative effects mediated through PPARγ. PGJ2 and other PPARγ agonists inhibit growth of breast and other cancer cell lines, and in most cancer cells, these effects are linked to apoptosis, enhanced expression of cyclin-dependent kinase inhibitors p21 and p27 and inhibition of G_1S-phase progression. PPARγ agonists in MCF-7 cells also induce proteasome-dependent degradation of both cyclin D1 and estrogen receptor alpha (ERα) (Qin et al., 2003). Houston and coworkers (2003) showed that PGJ2, troglitazone, and ciglitazone inhibit E2-stimulated cell proliferation of a leiomyoma-derived cell line and human primary leiomyoma cultures and also inhibit ER-mediated gene expression and protein expression. These results suggested that in uterine leiomyomas PPARγ activation is growth inhibitory and this inhibition is mediated at least in part by negative crosstalk between ER and PPARγ signaling pathways. However the underlying mechanism of the inhibitory ER- PPARγ crosstalk remains to be elucidated. Studies in this laboratory have also identified a novel class of synthetic PPARγ agonists, 1,1-Bis(3'indolyl)-1-(p-substitutedphenyl)methanes containing p-t-butyl (DIM-C-pPhtBu), and p-phenyl (DIM-C-pPhC6H5) groups, that induce responses in MCF-7 cells similar to those observed for PGJ2 (Qin et al., 2004).

The second objective of this study was to investigate the mechanisms underlying the inhibitory PPARγ-ER crosstalk using the *CAD* gene as a model and several PPARγ ligands including PGJ2, DIM-C-pPhtBu and DIM-C-pPhC6H5. PGJ2 inhibited E2-induced *CAD* gene expression and also downregulated E2-mediated transactivation of *CAD* gene promoter constructs and this was reversed by PPARγ antagonist T007 in MCF-7 cells. This suggests a possible inhibitory crosstalk between PPARγ and ER signaling pathways in breast cancer cells. In addition, DIM-C-pPhtBu and DIM-C-pPhC6H5 groups inhibited E2-mediated transactivation of *CAD* gene promoter constructs, however this effect was PPARγ-independent.

BODY

This project has been focused on the inhibitory effects of AhR agonists and PPARγ agonists on E2- induced expression of *CAD* gene in breast cancer cells. In this study we investigated the inhibitory effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on *CAD* gene expression (as described in Task 2) and we also studied the effects of PPARγ agonists such as PGJ2, and a new class of PPARγ agonists 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl) methanes on E2-induced *CAD* gene expression as described in task 1 of statement of work.

Previous results showed that E2 induces *CAD* gene expression in ZR-75 and MCF-7 breast cancer cells and this response is mediated through interaction of ERα/Sp1 with proximal GC-rich motifs (Khan et al., 2003) (Fig. 1). The *CAD* gene promoter construct pCAD1 (region -90 to +115) has 3 GC-rich elements and two E-boxes and hormone responsiveness was found to be associated with two upstream GC-rich sites.

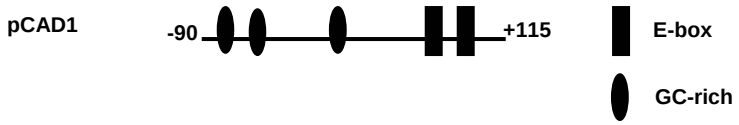


Figure 1. Schematic representation of *CAD* gene promoter construct pCAD1 (-90/+115).

TASK 2: Inhibition of hormone-induced *CAD* gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

TCDD inhibits hormone-induced activation of *CAD* gene expression and pCAD reporter gene activity

This study used the *CAD* gene as a model for investigating the mechanisms of inhibitory AhR-ERα crosstalk in which ERα/Sp1 was the hormone-activated transcription factor complex. We first investigated the effects of TCDD on E2-induced *CAD* gene expression. Results in Fig. 2A show that E2 induced *CAD* mRNA levels in ZR-75 cells and this response was inhibited by the antiestrogen ICI 182,780 and the AhR agonist TCDD. Results in Fig. 2B also show that treatment of MCF-7 cells with 10 nM E2 also induced *CAD* mRNA levels, whereas treatment with TCDD alone or in combination with E2 resulted in *CAD* mRNA levels similar to that observed in cells treated with DMSO (solvent control). These data demonstrate inhibitory AhR-ERα crosstalk associated with hormonal regulation of *CAD* gene expression in ER-positive ZR-75 and MCF-7 cells.

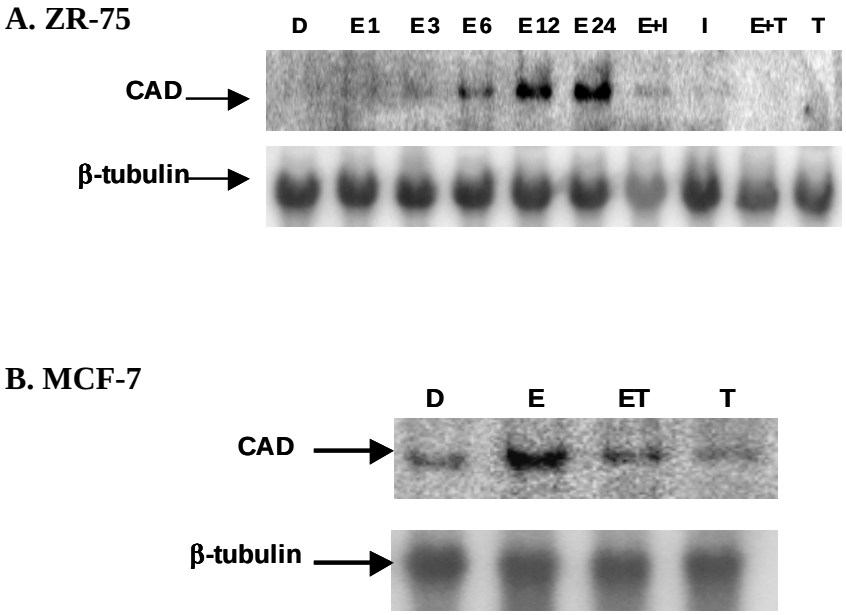


Figure 2. Regulation of *CAD* mRNA levels in ZR-75 and MCF-7 cells. ZR-75 cells [A] were treated with DMSO (D), 10 nM E2 (E) alone for 1-24 h, 1 μM ICI 182,780 (I), or 10 nM TCDD (T) alone for 12 h, or in combination with E2 (E+I or E+T) for 12 h. *CAD* mRNA levels were determined by Northern blot analysis. Using a similar approach, *CAD* mRNA levels were also determined in MCF-7 cells [B] treated with DMSO, 10 nM E2, 10 nM TCDD, or E2 plus TCDD for 6 h.

We then investigated inhibitory AhR-ERα crosstalk in ZR-75 and MCF-7 cells transfected with pCAD1 and constructs containing mutations in critical GC-rich sites and a potential iDRE motif containing a CACGC motif (Figs. 3 and 4). E2 induced transactivation in cells transfected with pCAD1 and pCAD2 which contains the E2-responsive -90 to +115 and -90 to +25 promoter inserts. After cotreatment with E2 plus TCDD, the induced luciferase activity was significantly decreased (Figs. 3A and 3B). In a similar set of experiments, ZR-75 cells were transfected with pCAD1 or pCAD2 and treated with DMSO, 10 nM E2, E2 plus ICI 182,780, or 1 μM ICI 182,780 alone (Figs. 3C and 3D). The results show that like TCDD, ICI 182,780 also inhibited E2-induced transactivation and the classical antiestrogen was a more effective inhibitor in the transient transfection studies. We also carried out a comparable set of experiments in MCF-7 cells transfected with pCAD1 or pCAD2 and treated with E2 alone or in the presence of 10 nM TCDD (Figs. 4A and 4B) or ICI 182,780 (Figs. 4C and 4D). The results were comparable to those observed in ZR-75 cells and both TCDD and ICI 182,780 inhibited E2-induced transactivation.

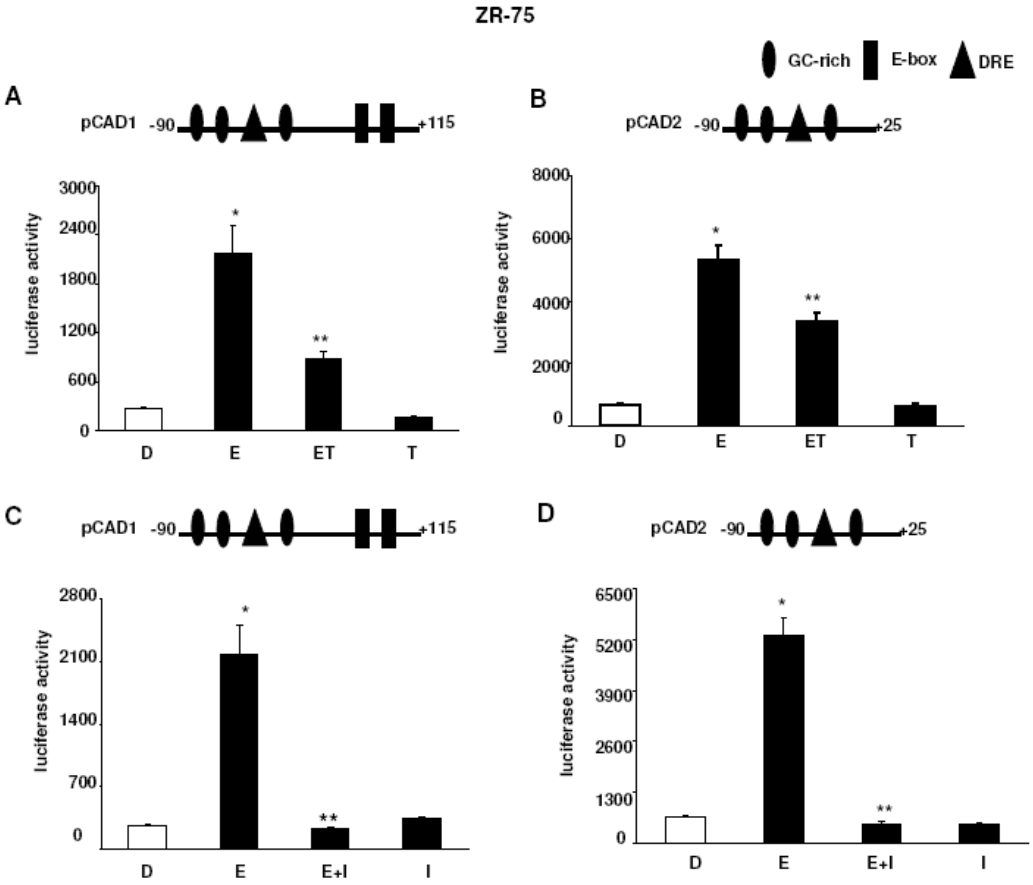


Figure 3. Regulation of *CAD* constructs in ZR-75 cells. ZR-75 cells were transfected with pCAD1 [A and C] or pCAD2 [B and D], treated with DMSO, 10 nM E2, 10 nM TCDD, or E2 plus TCDD [A and B] or DMSO, 10 nM E2, 1 μM ICI 182,780, or E2 plus ICI 182,780 [C and D] and luciferase activity was determined. Results are expressed as means ± SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition of this response by TCDD or ICI 182,780 (**) are indicated.

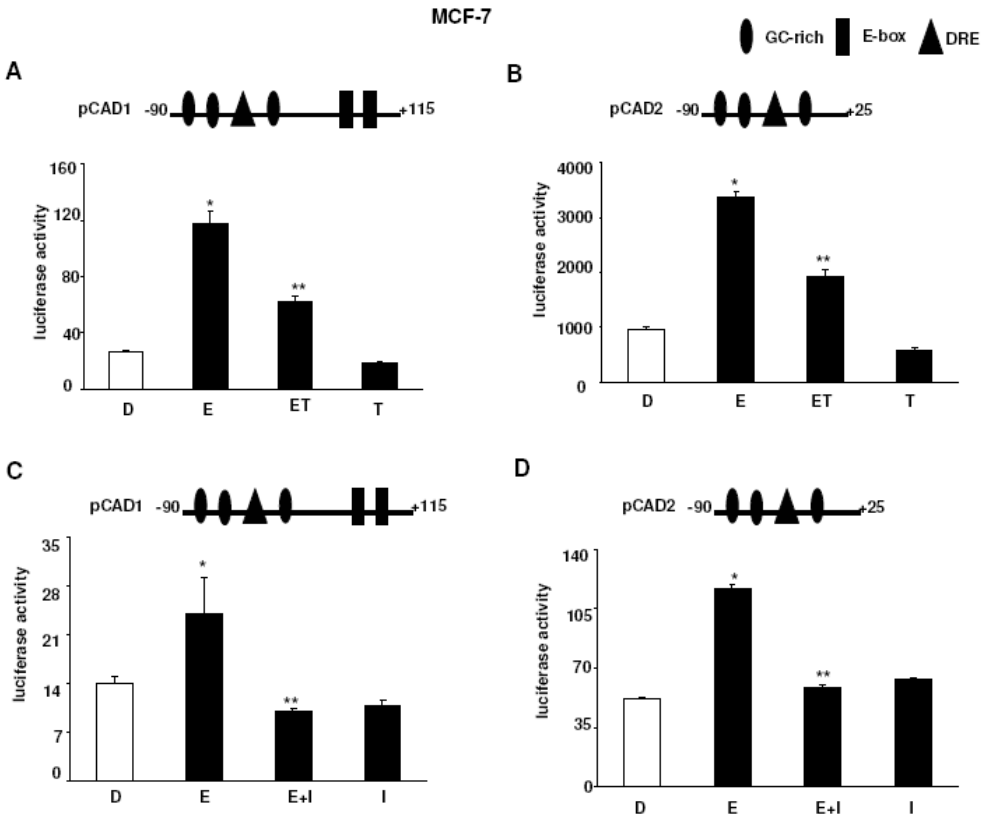


Figure 4. Regulation of *CAD* constructs in MCF-7 cells. Cells were transfected with pCAD1 [A and C] or pCAD2 [B and D], treated with DMSO, 10 nM E2, 10 nM TCDD, or E2 plus TCDD [A and B] or DMSO, 10 nM E2, 1 μM ICI 182,780, or E2 plus ICI 182,780 [C and D] and luciferase activity was determined. Results are expressed as means ± SE of three replicate determinations for each treatment group, and significant ($p < 0.05$) induction by E2 (*) or inhibition after cotreatment with TCDD or ICI 182,780 (**) are indicated.

We also carried out studies on inhibitory AhR-ERα crosstalk in ZR-75 and MCF-7 cells transfected with pCAD1 and constructs containing mutations in critical GC-rich sites and a potential iDRE motif (Fig. 5A). The upstream GC-rich sites #1 and #2 were previously identified as the major E2-responsive motifs in the *CAD* gene promoter (Khan et al, 2003), and the results in Figure 5B demonstrate that induction by E2 was increased in ZR-75 cells transfected with pCAD1m1 (mutated site #2), whereas in cells transfected with pCAD1m2, hormone-inducibility was not observed, indicating that GC-rich site #1 was sufficient for hormone inducibility. TCDD inhibited E2-induced transactivation in cells transfected with pCAD1m1. Inhibitory AhR-ERα crosstalk for cathepsin D, heat shock protein 27 and c-fos has been linked to direct interactions of the AhR complex with "inhibitory DREs" (iDREs) containing the core CACGC motif that binds the AhR complex (Krishnan et al., 1995; Porter et al., 2001). The *CAD* promoter also contains a CACGC motif at -45; however, E2 induced luciferase in ZR-75 cells transfected with pCAD1m3 (mutated DRE), and in cells cotreated with E2 plus TCDD, the induced response was significantly inhibited (Fig. 5C). Transfection of plasmids which contain the mutant iDRE and also mutations of GC-rich sites #1 (pCAD1m4) or #1 and #2 (pCAD1m5) resulted in loss of E2-responsiveness, and TCDD had no effect on this activity. These results suggest that the antiestrogenic activity of TCDD in ZR-75 cells was iDRE-independent; however, this would not preclude interaction of the AhR complex with the GC-rich *CAD* promoter since previous studies show that the AhR interacts with both ERα and Sp1 (Klinge et al., 1999; Kobayashi et al., 1996).

A parallel set of experiments were carried out in MCF-7 cells transfected with pCAD1, pCAD1m1 and pCAD1m2 (Fig. 5D), pCAD1, pCAD1m3, pCAD1m4 or pCAD1m5 (Fig. 5E). Although pCAD1m1 was hormone-inducible and this response was inhibited by cotreatment with TCDD (Fig. 5D), the magnitude of the induction response by E2 was significantly decreased suggesting a more important role for GC-rich site #2 in mediating activation by E2 in MCF-7 cells. Compared to results in ZR-75 cells (Fig. 5C), hormone induced transactivation was decreased in MCF-7 cells transfected with pCAD1m3 (Fig. 5D), suggesting that in MCF-7 cells, the CACGC sequence may also influence hormone-induced transactivation through the GC-rich site #2. This observation was not unprecedented since a previous study in MCF-7 cells showed that hormone-induced transactivation of a GC-rich motif in the cathepsin D promoter was also dependent on a proximal CACGC site (Wang et al., 1998). The pCAD constructs are clearly activated by E2 in ZR-75 and MCF-7 cells and ERα/Sp1 activation is a critical component of this process. However, results with the mutant constructs demonstrate, that cell context also plays a role in differential activation of specific promoter elements. Despite these differences in hormone-induced transactivation of wild-type and mutant *CAD* promoter constructs in ZR-75 and MCF-7 cells, inhibitory AhR-ERα crosstalk was observed for both gene and reporter gene expression in both cell lines.

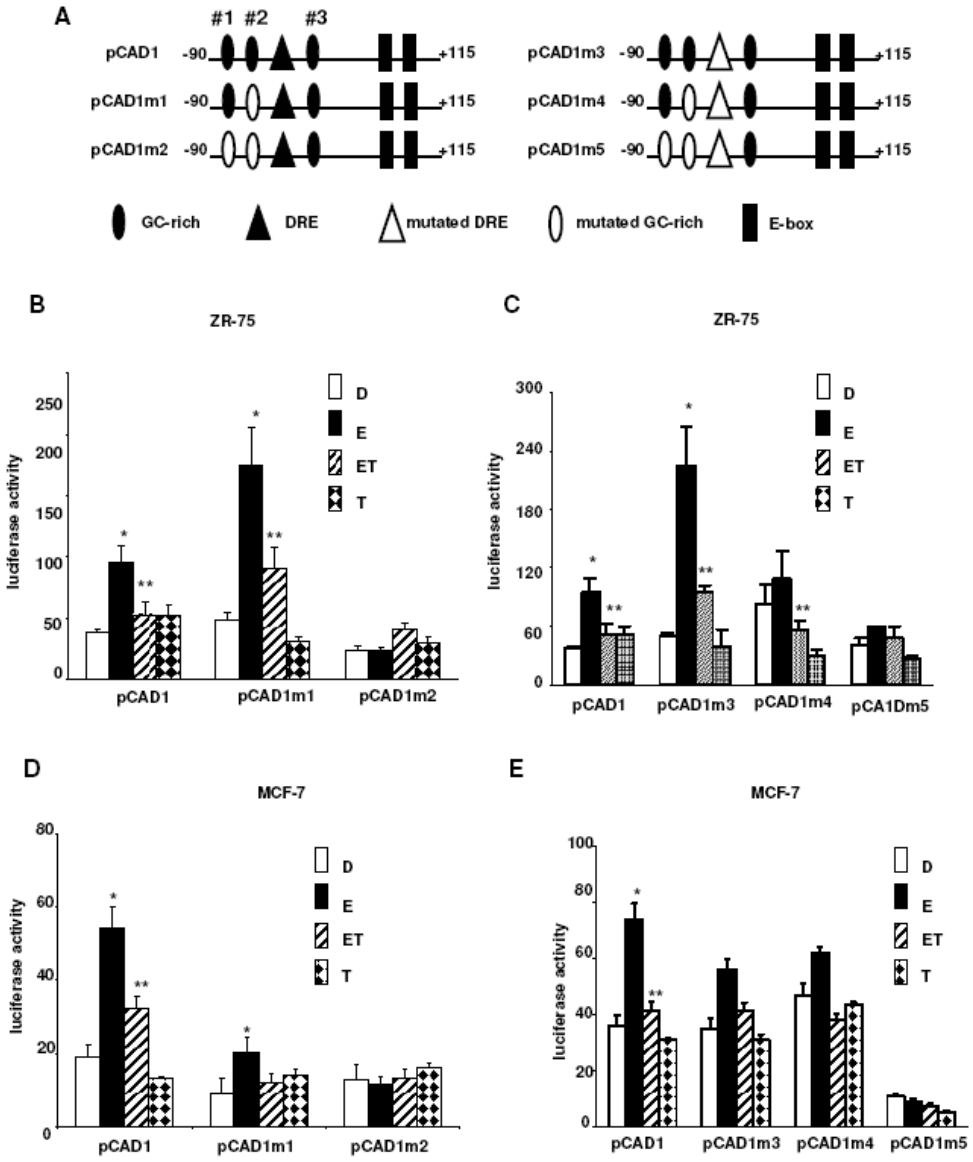


Figure 5. Inhibitory AhR-ERα crosstalk in cells treated with wild-type or mutant *CAD* constructs. [A] Summary of *CAD* constructs and their *cis* elements. Transfection of wild-type and mutant *CAD* constructs in ZR-75 [B and C] and MCF-7 [D and E] cells. Cells were transfected with the constructs, treated with DMSO, 10 nM E2, 10 nM TCDD, or E2 plus TCDD, and luciferase activity determined was determined. Results are expressed as means ± SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition by E2 plus TCDD (**) are indicated.

Characterization of CFP and YFP fusion proteins used in florescence resonance energy transfer (FRET)

FRET has been used to study interactions of nuclear receptors and co-regulatory proteins or peptides in living cells (Llopis et al., 2000; Tamrazi et al., 2002; Weatherman et al., 2002; Bai and Giguere, 2003), and using YFP/CFP chimeras of ERα and Sp1, ligand-induced interactions of ERα with Sp1 in MCF-7 cells have been reported (Kim et al., 2005). Figure 6A summarizes the YFP/CFP chimeras used in this study in MCF-7 cells. ZR-75 cells exhibit lower transfection efficiencies and were not used for the FRET studies. In a previous report (Kim et al., 2005), it was shown that the YFP/CFP-ERα and Sp1 chimeras were functional in transactivation assays. Figure 6B summarizes the effects of DMSO, E2, and E2 plus TCDD on distribution of transfected YFP-AhR in MCF-7 and COS-1 cells. In MCF-7 cells treated with DMSO and E2, the AhR was detected in the cytosolic and nuclear fractions, whereas after treatment with E2 plus TCDD or TCDD alone, the receptor was localized exclusively in the nucleus and exhibited a punctate staining pattern. In a parallel experiment with COS-1 cells that do not express ER or AhR, the transfected YFP-AhR was both cytosolic/perinuclear and nuclear in cells treated with DMSO or E2, whereas E2 plus TCDD or TCDD alone induced formation of a nuclear AhR complex as observed in MCF-7 cells. The functionality of the YFP-AhR chimera was also investigated in COS-1 cells treated with DMSO or 10 nM TCDD and transfected with $pDRE_3$ which contains three tandem consensus DREs linked to firefly luciferase (Figure 6C). In the absence of YFP-AhR, TCDD did not induce luciferase activity; however, induction by TCDD was observed after cotransfection of YFP-AhR indicating that the chimeric YFP-AhR protein was functional.

Ligand-dependent AhR activation inhibits ERα-Sp1 interactions as determined by FRET

Ligand activation of the AhR complex inhibits induction of ERα/Sp1-dependent activation of *CAD* gene/gene promoter expression in MCF-7 and ZR-75 cells (Figs. 2 - 4), and the effects of AhR and ERα ligands on interactions of CFP-ERα and YFP-AhR in living cells were determined by FRET in MCF-7 and COS-1 cells (Fig. 7). In solvent (DMSO)-treated MCF-7 cells transfected with YFP-AhR and CFP-ER, both receptors were primarily localized in the nucleus and this was also observed in cells treated with 10 nM E2 , 10 nM TCDD, or E2 plus TCDD (Fig. 7A). Cells were pretreated with TCDD for 10 min and then treated with E2 (alone or in combination) for an additional 8 min. A punctate nuclear pattern was observed in all the ligand treated groups and was most pronounced in cells treated with E2 plus TCDD. Excitation of CFP-ER at 410 nm and emission at 488 illustrates the blue fluorescent emission of the nuclear CFP-ER. The yellow fluorescence was detected in the FRET channel at 525 nm and this represents the CFP-YFP interaction and energy transfer. The emission intensities in the FRET channel were enhanced in the treated cells. The results in Figure 7A also quantitate the FRET efficiencies in the various treatment groups, and there was a significant increase in FRET efficiencies in cells treated with E2, TCDD and E2 plus TCDD. The overlay of the CFP and FRET signals shown in Figure 7A confirm the enhanced emission observed in the FRET channel for the treatment groups.

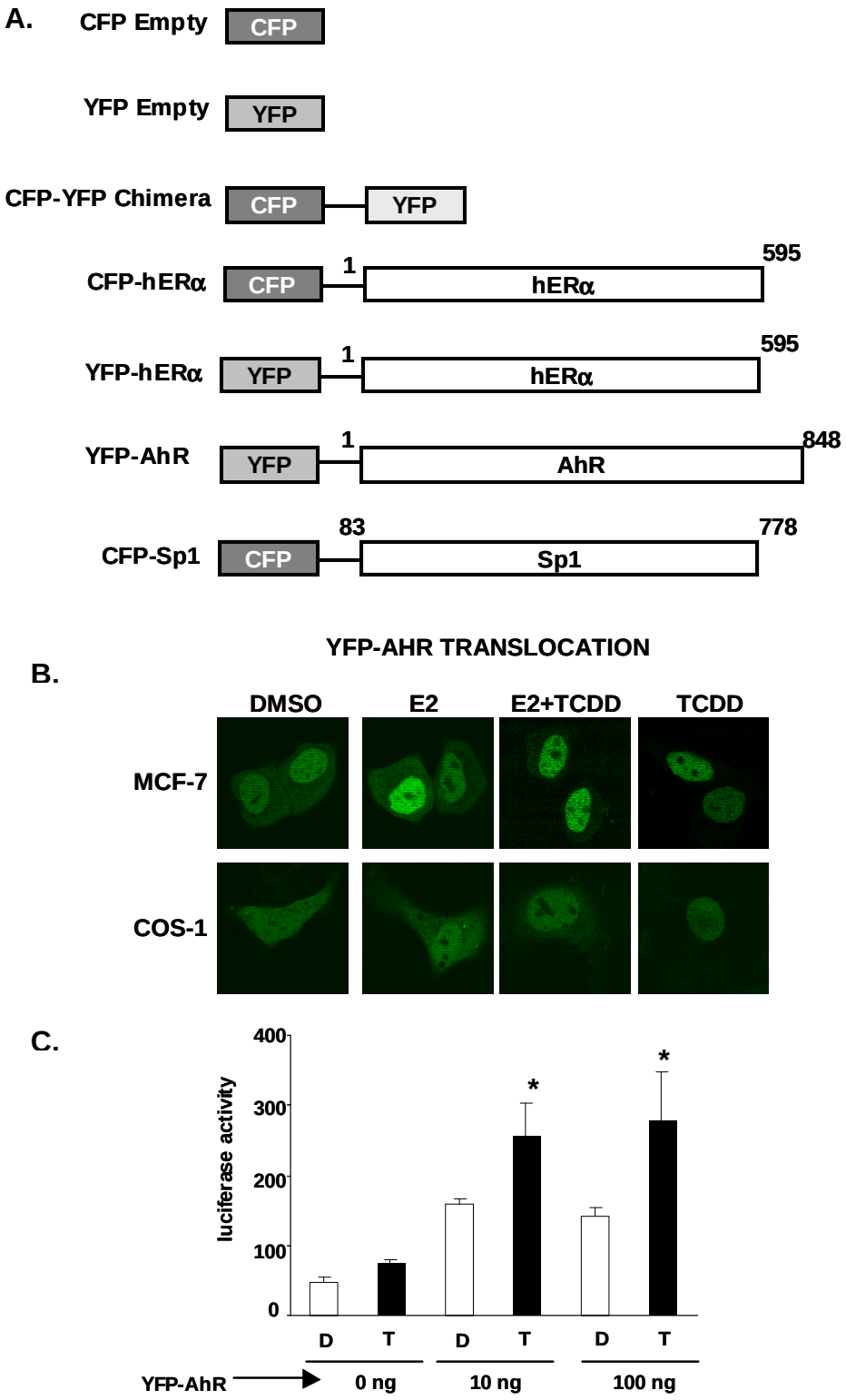


Figure 6. Chimeric expression plasmids and activity of YFP-AhR. [A] Summary of chimeric constructs used for FRET studies. [B] Ligand-dependent subcellular trafficking of YFP-AhR. MCF-7 or COS-1 cells were transfected with YFP-AhR, treated with

DMSO, 10 nM E2, 10 nM TCDD, or E2 plus TCDD, and localization of YFP-AhR was determined as described in the Materials and Methods. [C] Ah-responsiveness of COS-1 cells transfected with YFP-AhR. COS-1 cells were transfected with pDRE3 and different amounts of YFP-AhR expression plasmid, treated with DMSO or 10 nM TCDD, and luciferase activity was determined. Results are expressed as means ± SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by TCDD is indicated (*).

A parallel set of experiments was also carried out in COS-1 cells that do not express endogenous AhR or ERα. In COS-1 cells transfected with CFP-ER and YFP-AhR (Fig. 7B), the results of excitation/emission studies were similar to those observed in MCF-7 cells and FRET efficiencies were significantly increased in COS-1 cells treated with E2, TCDD and E2 plus TCDD (Fig. 7B).

Direct interactions of chimeric Sp1 and AhR proteins were not observed in the FRET assay, and this is not unexpected due to the high molecular weights of these proteins which preclude adequate distance (1 - 10 nm) between the fluorophores to observe energy transfer. We therefore investigated the effects of the liganded AhR complex on hormone dependent activation of ERα/Sp1 in MCF-7 cells which express endogenous AhR. Cells were transfected with CFP-Sp1 and YFP-ERα and treated with solvent (DMSO) control, E2, TCDD or E2 plus TCDD. Cells were pretreated with TCDD for 10 min prior to addition of E2 for 8 min (Fig. 8A). Cells treated with DMSO or TCDD exhibit low FRET efficiencies, whereas after treatment with E2, there was a significant increase in the FRET signal. This ligand-dependent increase was consistent with our recent FRET study showing ERα-Sp1 interactions in breast cancer cells (Kim et al, 2005). However, the intensity of the E2-induced FRET emission is significantly decreased in cells treated with E2 plus TCDD, and quantitation of the FRET efficiencies summarized in Figures 8B confirms this observation. These data indicate that the liganded AhR complex induces a rapid change in ERα/Sp1 interactions which correlates with the observed inhibitory AhR-ERα crosstalk on the *CAD* gene/gene promoter (Figs. 2 - 5). We further investigated ER-AhR interactions in MCF-7 cells treated with DMSO, E2, TCDD, E2 plus TCDD and transfected with FLAG-AhR. Cell lysates were immunoprecipitated with nonspecific IgG or FLAG antibodies and analyzed for ERα by Western blot analysis (Fig. 9A). ERα was detected in IgG precipitates, and the lower levels were observed in the E2 plus TCDD treatment group. Interactions of ERα with the AhR were determined in the FLAG antibody immunoprecipitates in which higher levels of ERα were observed in the TCDD and E2 plus TCDD treatment groups. These results were consistent with the enhanced AhR-ERα interactions observed by FRET in MCF-7 and COS-1 cells treated with TCDD and E2 plus TCDD (Fig. 7). Results in Figure 8B show that TCDD induced transactivation (compared to DMSO), thus confirming that the FLAG-AhR chimera was functional.

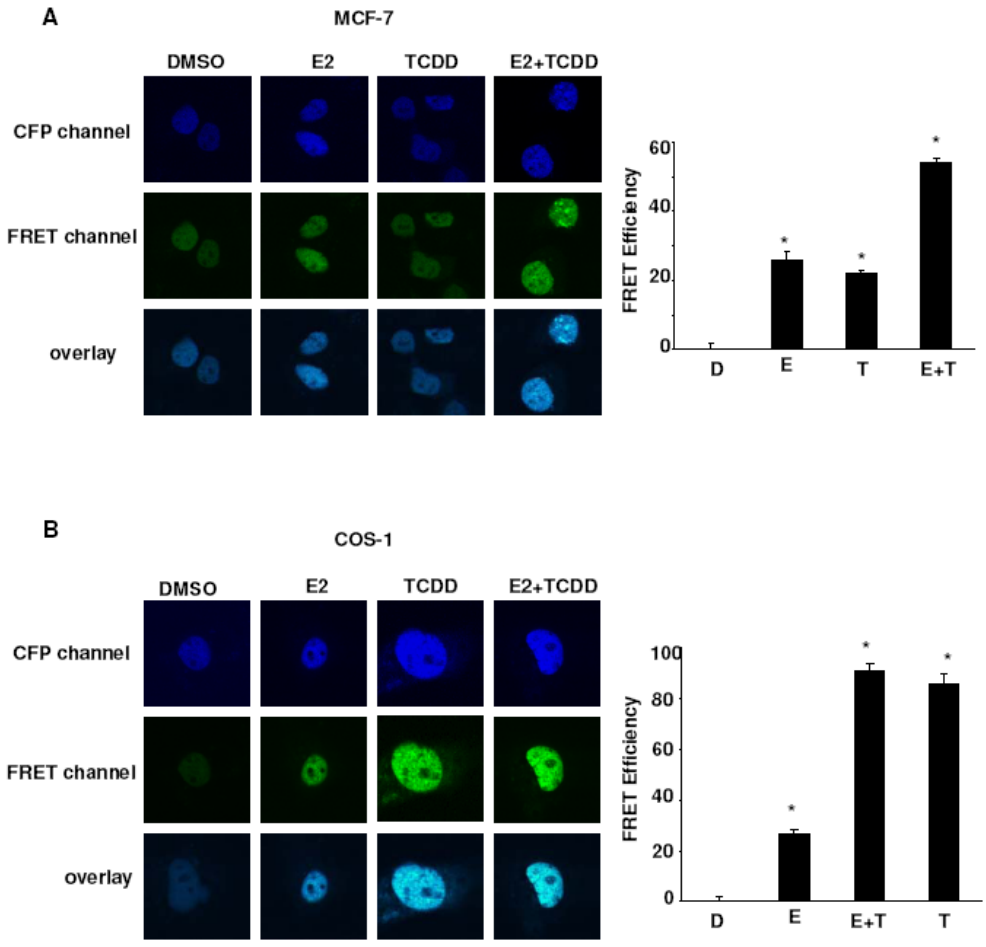


Figure 7. Ligand-dependent interactions of YFP-AhR and CFP-ERα. MCF-7 [A] and COS-1 [B] cells were transfected with YFP-AhR and CFP-ERα, treated with DMSO, 10 nM E2, 10 nM TCDD, and TCDD plus E2 where TCDD was added 10 min prior to treatment with E2. Representative FRET images in each treatment group were acquired after 8 min and FRET efficiencies in MCF-7 and COS-1 cells transfected with YFP-AhR and CFP-ERα were acquired from images taken from 8 - 18 min after treatment. For each treatment group, 10 - 15 images were acquired and each image contained 1 - 5 cells which were subsequently analyzed. Background signals from the images were subtracted, and significant ($p < 0.05$) induction of FRET efficiency in the various treatment groups is indicated by an asterisk.

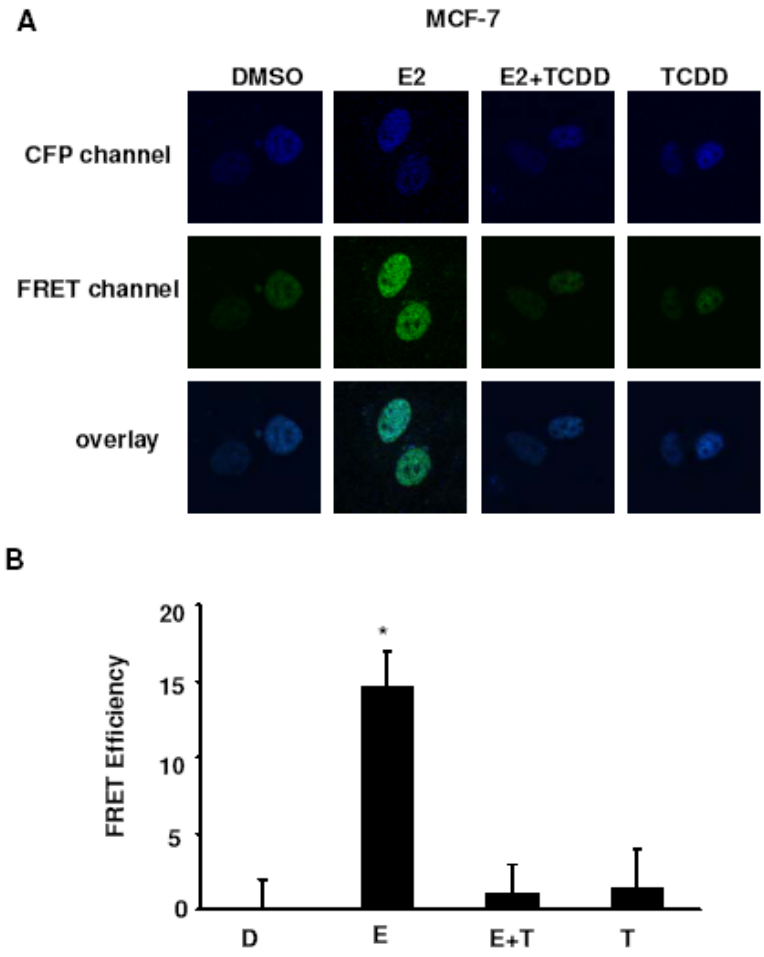


Figure 8. Ligand-dependent interactions between YFP-ER and CFP-Sp1, treated with DMSO, 10 nM E2, 10 nM TCDD, or TCDD plus E2 where TCDD was added 10 min prior to treatment with E2. Representative images in each treatment group were acquired after 8 min and FRET efficiencies in the various groups were determined 8 – 18 min after treatment. For each treatment group, 10 - 15 images were acquired and each image contained 1 - 5 cells which were analyzed for FRET efficiency by subtracting background signals. Significant ($p < 0.05$) induction of FRET efficiency by E2 (*) and inhibition of this response by cotreatment with TCDD (**) are indicated.

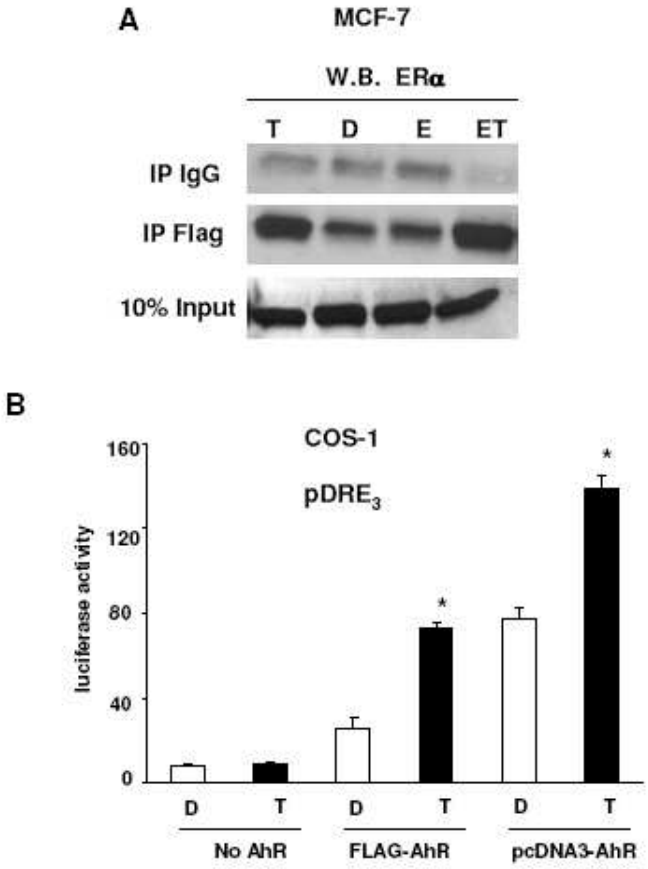


Figure 9. FLAG-AhR interaction with ERα. [A] Coimmunoprecipitation experiments. MCF-7 cells were transfected with FLAG-AhR expression plasmid, and cells were treated with DMSO, 10 nM TCDD, 10 nM E2, or TCDD plus E2 for 30 min, and whole cell lysates were immunoprecipitated with IgG or FLAG antibodies and then analyzed by Western blot assays for ERα. [B] Ah-responsiveness of FLAG-AhR. COS-1 cells were transfected with pDRE3 and FLAG-AhR or AhR expression (in pcDNA3) plasmids, cells were then treated with DMSO or TCDD and luciferase activity was determined. Results are means ± SE for three replicate determinations for each treatment group and significant ($p < 0.05$) induction by TCDD is indicated by an asterisk.

There is also evidence that proteasome-dependent degradation of ERα in breast cancer cells cotreated with E2 plus TCDD may result in limiting levels of ERα and thereby inhibit expression of E2-responsive genes (Wormke et al., 2003). Therefore we investigated the effects of proteasome inhibitor MG132 on *CAD* mRNA levels in presence of different ligands. Results in Figure 10 show that E2 induced *CAD* mRNA levels in ZR-75 cells and this was downregulated in cells co-treated with E2+TCDD. However, in cells pre-treated with MG132, *CAD* mRNA levels were decreased in all the treatments including the control. Thus in this study it was not possible to determine whether the low levels of ERα in cells cotreated with E2 plus TCDD were limiting since the proteasome inhibitor MG132 itself directly decreased *CAD* mRNA levels (Figure 10). Thus, it is possible that decreased and possibly limiting ERα levels in MCF-7 and ZR-75 cells cotreated with E2 plus TCDD may contribute to the antiestrogenic effects of the latter compound.

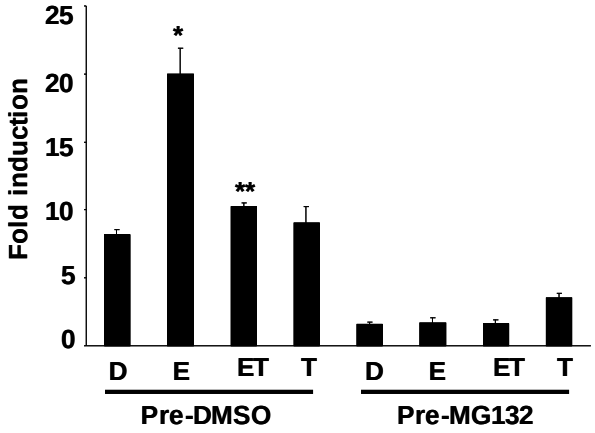


Figure 10. Effects of proteasome inhibitor MG132 on *CAD* mRNA levels in ZR-75 cells. ZR-75 cells were pretreated with DMSO (D) or 10 μM MG132 before treating with DMSO, 10 nM E2 (E), 10 nM TCDD (T) or E+T for 12 h and *CAD* mRNA levels were determined by Real-time PCR analysis. Significant ($p < 0.05$) induction by E2 (*) and inhibition of this response by TCDD (**) are indicated.

Analysis of ERα, AhR and other transcription Factor interactions with the *CAD* and *CYP1A1* gene promoters in MCF-7 and ZR-75 Cells

Results of the FRET experiments suggest that the AhR complex either forms a transcriptionally-inactive AhR:ER/Sp1 complex where the AhR suppresses ER/Sp1 action or the AhR competitively dissociates ERα from interactions with Sp1 or both pathways are operative. AhR-dependent dissociation of ERα from Sp1 is supported, in part, by recent studies showing that treatment of cells with TCDD alone or in combination with E2 recruits the ER/AhR complex

to promoters of Ah-responsive genes such as *CYP1A1* (Beischlag and Perdew, 2005; Matthews et al, 2005). We therefore investigated simultaneous interactions of AhR/Arnt and ERα on the endogenous *CAD* and *CYP1A1* gene promoters (Fig. 11A) using a ChIP assay. Initial studies examined interactions of ERα, Sp1, AhR and Arnt with the *CAD* gene promoter in ZR-75 cells after treatment with DMSO, 10 nM E2, 10 nM TCDD, and E2 plus TCDD for 1 h (Fig. 11B). There was evidence that all of these transcription factors were associated with the E2-responsive (GC-rich) region of the *CAD* promoter in the solvent (DMSO)-treated group and similar results were obtained in MCF-7 cells. Arnt and Sp1 levels exhibited minimal changes in band intensities in the various treatment groups. The ERα band increased and decreased in cells treated with TCDD and E2 plus TCDD, respectively, and in cells treated with TCDD, there was a decrease in AhR interaction with the *CAD* promoter. These results show some treatment related differences at one specific time point (1 h) and, in order to more accurately define AhR/ERα interaction with the *CAD* promoter during conditions of inhibitory AhR-ERα/Sp1 crosstalk (i.e. E2 plus TCDD), we determined the time-dependent interactions of transcription factors with the *CAD* promoter in ZR-75 (Fig. 11C) and MCF-7 (Fig. 11D) cells cotreated with E2 plus TCDD. In ZR-75 cells, band intensities associated with Arnt and Sp1 were similar at all time points (0, 15, 60 or 120 min), whereas after 60 or 120 min, there was increase in bands associated with the AhR and a decrease in the ERα band. These results are similar to the 60 min ChIP assay results in the E2 plus TCDD treatment group (Fig. 11B). As a positive control for this experiment in ZR-75 cells, we also showed that treatment with TCDD plus E2 recruited AhR, Arnt and ERα to the Ah-responsive region of the *CYP1A1* promoter (Fig. 11C). The time dependent recruitment of AhR, ERα, Arnt and Sp1 to the *CAD* and *CYP1A1* promoters were also determined in MCF-7 cells treated with E2 + TCDD (Fig. 11D). In MCF-7 cells, only minimal changes in ERα and AhR band intensities were observed after 120 min; however, recruitment of AhR, Arnt and ERα to the *CYP1A1* promoter was comparable in both cell lines (Figs. 11C and 11D). As a positive control for these interactions, we also compared hormone-induced changes in the interaction of ERα with the *CAD* promoter and the region of the *pS2* gene promoter containing a functional ERE The results (Fig. 11E) were obtained using two ERα antibodies and show that treatment with E2 strongly enhanced ERα interactions with the *pS2* promoter as previously described (Krieg et al., 2004; Acevedo et al., 2004; Metivier et al., 2003; Shao et al., 2002). In contrast, ERα (and Sp1) are constitutively bound to the *CAD* gene promoter in MCF-7 and ZR-75 cells and treatment with E2 does not markedly affect ERα binding to the promoter. We have observed constitutive binding of ERα and Sp1 to GC-rich promoters of other E2-responsive genes as well. Figure 11F is a control experiment showing that the transcription factor TFIIB binds the *GAPDH* promoter but not exon 1 of the *CNAP* gene as previously reported. Results of the ChIP assay suggest that AhR-ERα/Sp1 crosstalk in cells cotreated with E2 plus TCDD involves ligand-induced disruption of ERα/Sp1 by the AhR, and this is accompanied by slightly decreased ERα interactions with the *CAD* gene promoter only in ZR-75 cells, whereas ERα is recruited to the *CYP1A1* promoter in both cell lines. It was also apparent from replicate experiments that full dissociation of ERα from the *CAD* gene promoter in ZR-75 cells was not observed, suggesting that AhR may act by both suppressing ERα/Sp1 action and sequestering ERα.

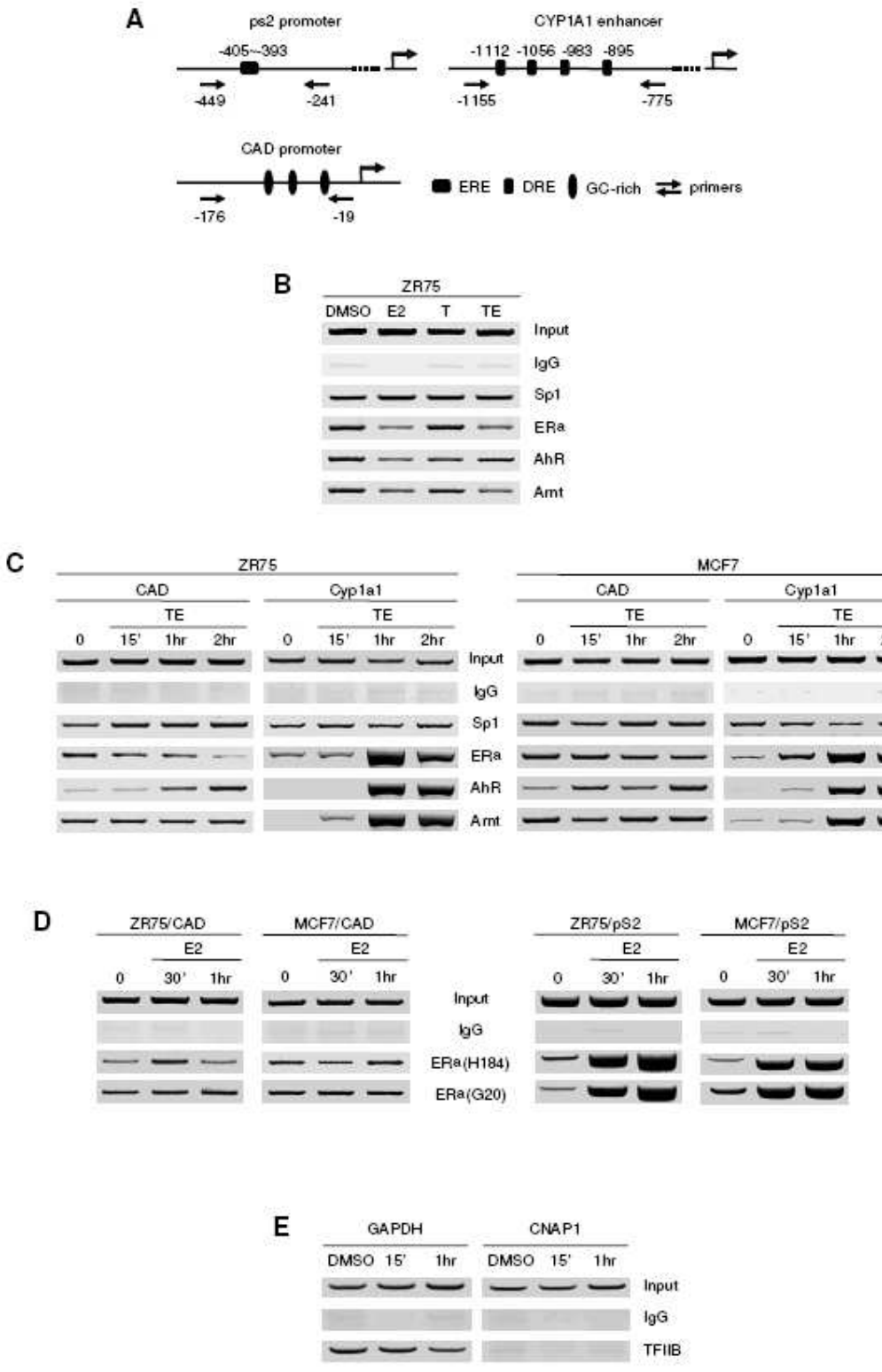


Figure 11. Chromatin immunoprecipitation assay. [A] promoter and primers. The E2-responsive GC-rich and ERE sites on the *CAD* and *pS2* promoters and Ah-responsive sites (DREs) on the human *CYP1A1* promoter and primer locations are indicated. ChIP

assay on the *CAD* [B] and *CAD/CYP1A1* [C] in ZR-75 cells and *CAD/CYP1A1* [D] in MCF-7 cells. Cells were treated with various reagents for 1 h [B] or 15 min, 1 or 2 h, and analysis of proteins interacting with promoters of the *CAD* and *CYP1A1* genes were determined. [E] Interactions of ERα with the *CAD* and *pS2* gene promoters. MCF-7 or ZR-75 cells were treated with E2 for 30 or 60 min, and analysis of proteins interacting with the E2-responsive regions of both promoters was determined by ChIP. [F] Control binding of TFIIB. The control ChIP assay illustrates binding of TFIIB to the GAPDH promoter but not exon 1 of the *CNAP1* gene (negative control).

Results of this study suggest a novel inhibitory mechanism where the liganded AhR disrupts formation of a transcriptionally active ERα/Sp1 complex which remains bound to the E2-responsive region of the *CAD* gene promoter. ERα/Sp1 and the AhR complexes are associated with the *CAD* promoter in the presence or absence of E2, TCDD or E2 plus TCDD as determined in a ChIP assay. FRET studies clearly demonstrate that the liganded AhR disrupts interactions of ERα and Sp1 and only the combination of both ChIP and FRET assay provides the necessary insights on this mechanism. These results highlight some of the early events (≤ 2 hr) associated with AhR-dependent inhibition of ERα/Sp1 action. It is also likely that decreased gene expression is accompanied by redistribution of coactivators/corepressor interacting with the AhR/ERα/Sp1 complex and these are currently being investigated. Future studies will also determine if the mechanisms of inhibitory crosstalk observed for the *CAD* gene promoter are similar to those for other E2-responsive genes regulated by ERα/Sp proteins.

TASK 1: Inhibition of E2-induced *CAD* gene expression by PPARγ ligands

PGJ2 inhibits hormone-induced activation of *CAD* gene expression and pCAD reporter gene activity

PPARγ is widely expressed in multiple tumors and cell lines, and this receptor has also become a target for developing new anticancer drugs that will take advantage of the antiproliferative and proapoptotic effects mediated through PPARγ. We initially investigated the effects of the PPARγ agonist PGJ2 on hormone-induced *CAD* gene expression and *CAD* gene promoter constructs. MCF-7 cells were treated with 10 nM E2, 10 μM PGJ2, 20 μM PGJ2 or E2+PGJ2 for 12 h and mRNA levels were determined (Fig. 12). Significant induction by E2 was observed after 12 h and this was significantly down regulated by PGJ2.

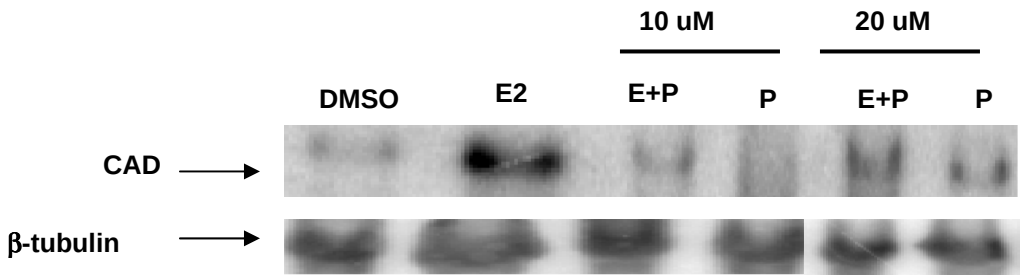
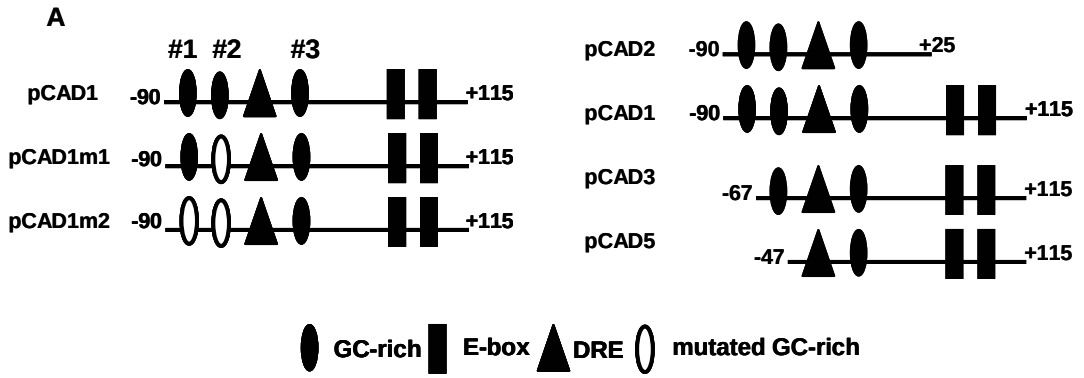
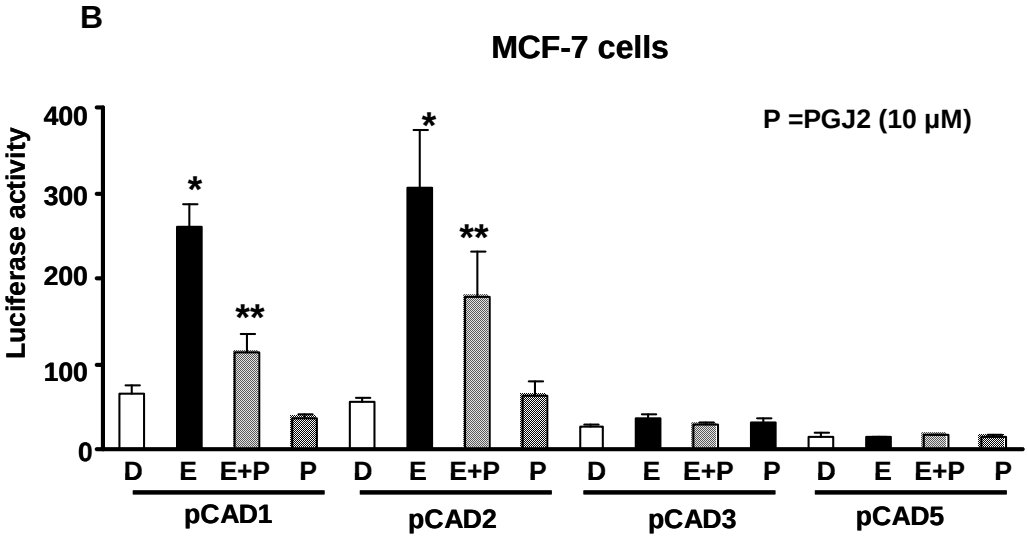


Figure 12. Effect of the PPARγ agonist PGJ2 on E2-induced *CAD* gene expression in ZR-75 cells. The cells were treated DMSO, 10 nM E2 (E), 10 μM PGJ2 (P) or E+P for 12 h. Cell extracts were obtained, and total RNA was isolated and subjected to Northern analysis. The intensity values were normalized to the values of β-tubulin mRNA.

The inhibitory effect of PGJ2 on E2-induced transactivation was also investigated in MCF-7 (Fig. 13) cells transfected with constructs containing *CAD* gene promoter inserts. E2 induced transactivation in cells transfected with pCAD1 (-90/+115) and pCAD2 (-90/+25) constructs and after cotreatment with E2 plus PGJ2, the hormone-induced response (luciferase activity) was significantly decreased. Constructs containing deletions of proximal GC-rich sites [pCAD3 (-67/+115) and pCAD5 (-47/+115)] were not induced by E2. Also the constructs containing point mutations in the functional GC-rich site (#1) in pCAD1m1 was downregulated by PGJ2 whereas pCAD1m2 which contains point mutations in both upstream GC-rich sites was not affected by E2 or PGJ2. Moreover, the downregulation of E2-mediated transactivation of *CAD* gene promoter constructs in transient transfection assays was reversed by PPARγ antagonist N-(4v-aminopyridyl)-2-chloro-5-nitrobenzamide (T007) in MCF-7 cells suggesting that the observed inhibition of E2-induced *CAD* gene expression by PGJ2 is PPARγ-dependent (Fig. 14).





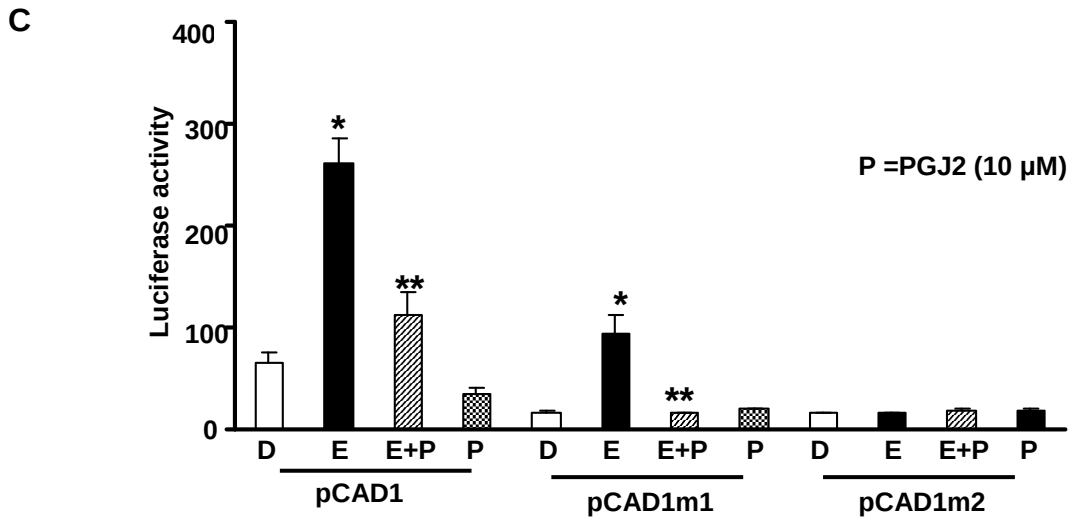


Figure 13. Inhibition of estrogen-induced transactivation in cells transfected with pCAD constructs and treated with PGJ2 in MCF-7 cells. A. Summary of *CAD* constructs and their *cis* elements. Deletion (B) & mutatuion (C) analysis of the CAD promoter in MCF-7 cells. MCF-7 cells were transfected with pCAD promoter constructs, treated with DMSO (D), 10 nM E2, 10 μM PGJ2 plus E2 or PGJ2 alone and luciferase activities were determined. Results are expressed as means ± SD for at least three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition by E2 plus PGJ2 (**) are indicated.

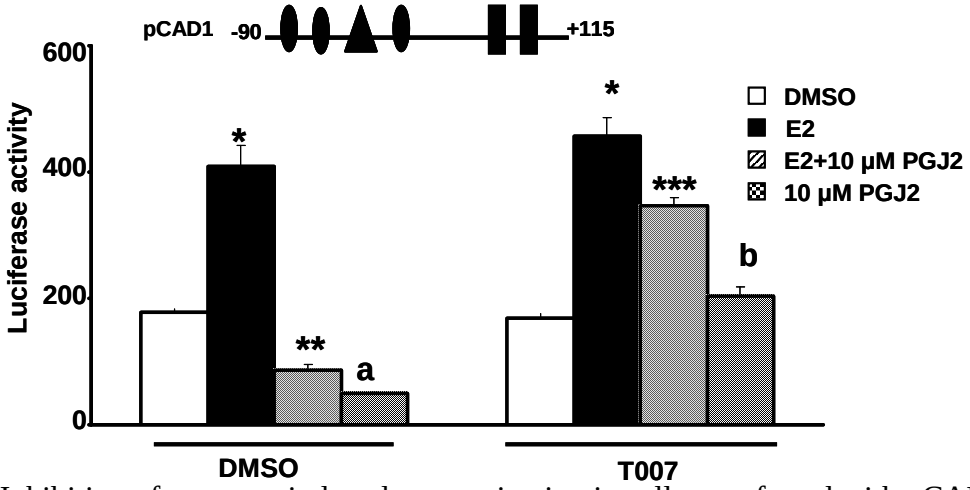


Figure 14. Inhibition of estrogen-induced transactivation in cells transfected with pCAD constructs and treated with PGJ2 in MCF-7 cells. MCF-7 cells were transfected with pCAD constructs, treated with DMSO (D), 10 nM E2, 10 μM PGJ2 plus E2 or PGJ2 alone in presence or absence of PPARγ antagonist T007 (10μM) and luciferase activities were determined. Results are expressed as means ± SD for at least three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition by E2 plus PGJ2 (**) are indicated. Also significant inhibition of basal activity by PGJ2 (a) and its reversal by T007 (b) are indicated.

DIM-C-pPhtBu and DIM-C-pPhC6H5 inhibits hormone-induced activation of *CAD* gene expression and reporter gene activity in cells transfected with pCAD constructs

1,1-Bis(3'indolyl)-1-(p-substitutedphenyl)methanes containing p-t-butyl (DIM-C-pPhtBu), and p-phenyl (DIM-C-pPhC6H5) groups (Fig. 15) have been investigated as a new class of PPARγ agonists and these compounds inhibit breast cancer growth (Qin et al, 2004). Here we investigated the effects of DIM-C-pPhtBu (# 4) and DIM-C-pPhC6H5 (# 9) on E2-mediated transactivation of pCAD constructs in MCF-7 cells (Fig. 16). E2 induced transactivation in cells transfected with pCAD1 (-90/+115) and pCAD2 (-90/+25) construct and after cotreatment with E2 plus DIM-C-pPhtBu (# 4) or E2 plus DIM-C-pPhC6H5 (# 9), the induced response (luciferase activity) was significantly decreased. However this inhibitory effect was not reversed by PPARγ antagonists T007 and GW9662 suggesting that the inhibition of hormone-induced *CAD* gene promoter constructs by DIM-C-pPhtBu (# 4) and DIM-C-pPhC6H5 (# 9) is PPARγ-independent (Fig. 17). Fig. 17C also shows that knockdown of PPARγ by RNA interference (iPPARγ) did not affect the inhibition of E2-induced luciferse activity by the C-DIM compounds further confirming that this response was PPARγ-independent. The mechanistic differences between PGJ2 and PPARγ-active C-DIM compounds were surprising and current studies are investigating the PPARγ agonists structure-dependent effects on CAD and other key hormone-induced genes in breast cancer cells.

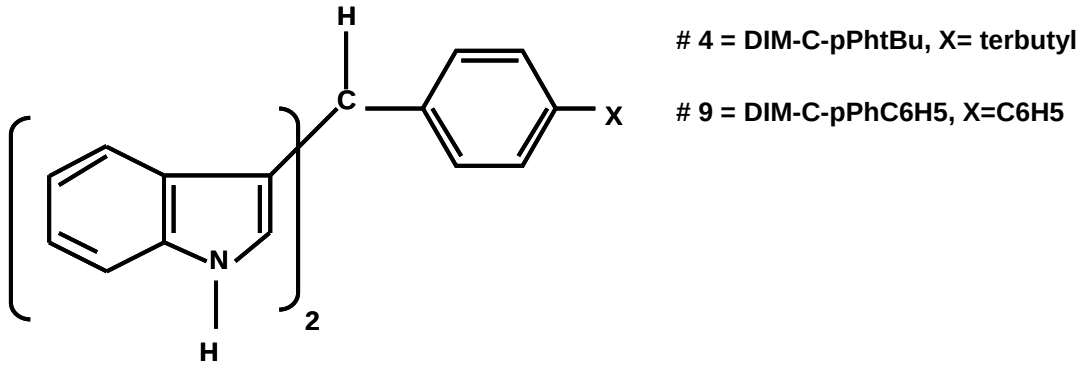


Figure 15. Structure of C-DIMs.

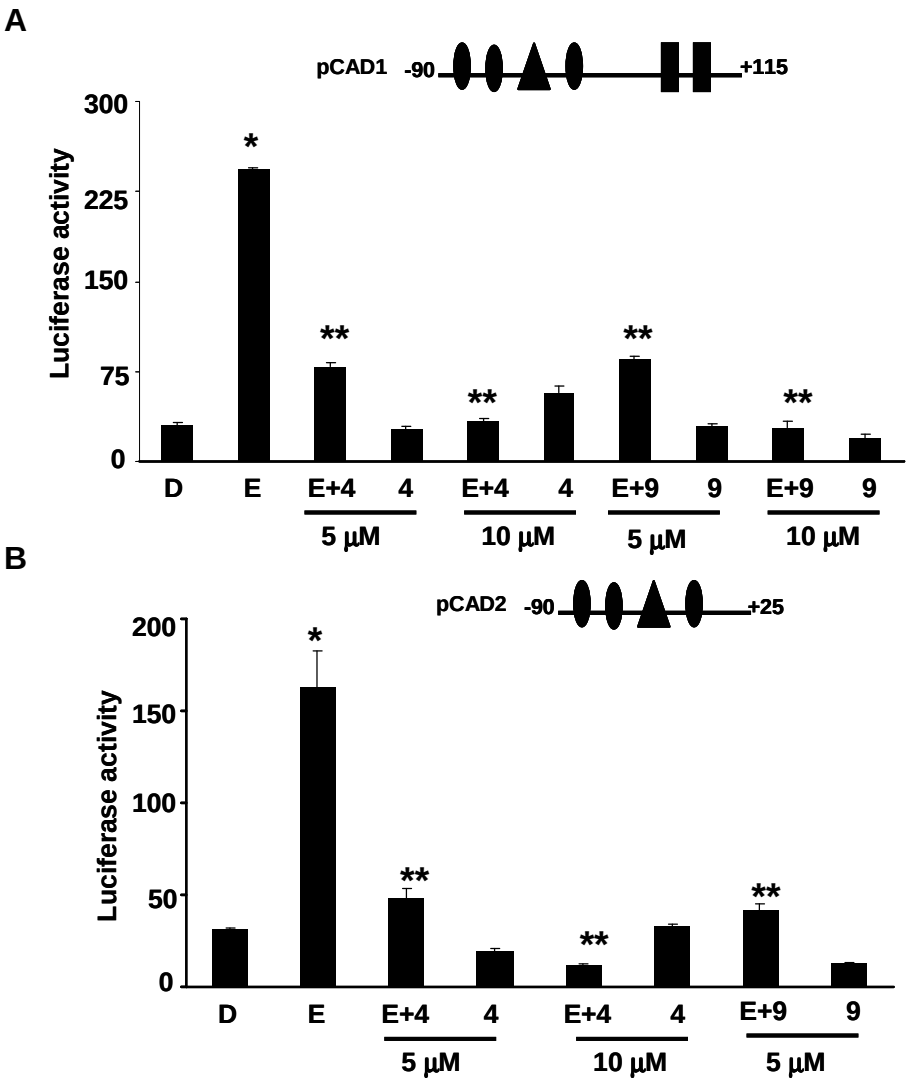
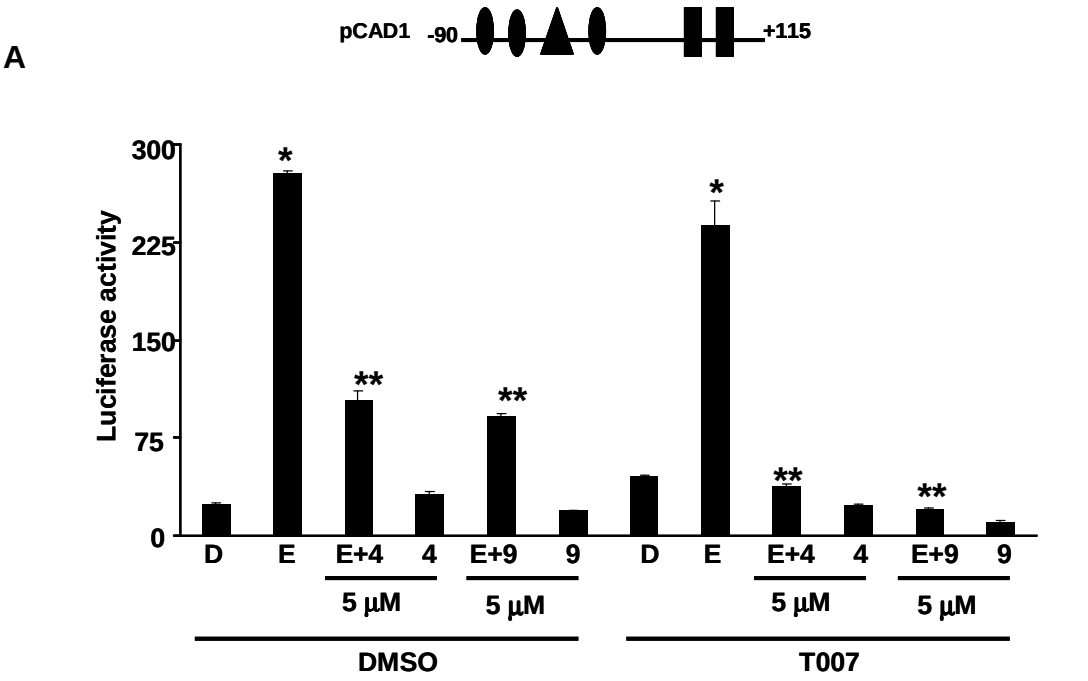
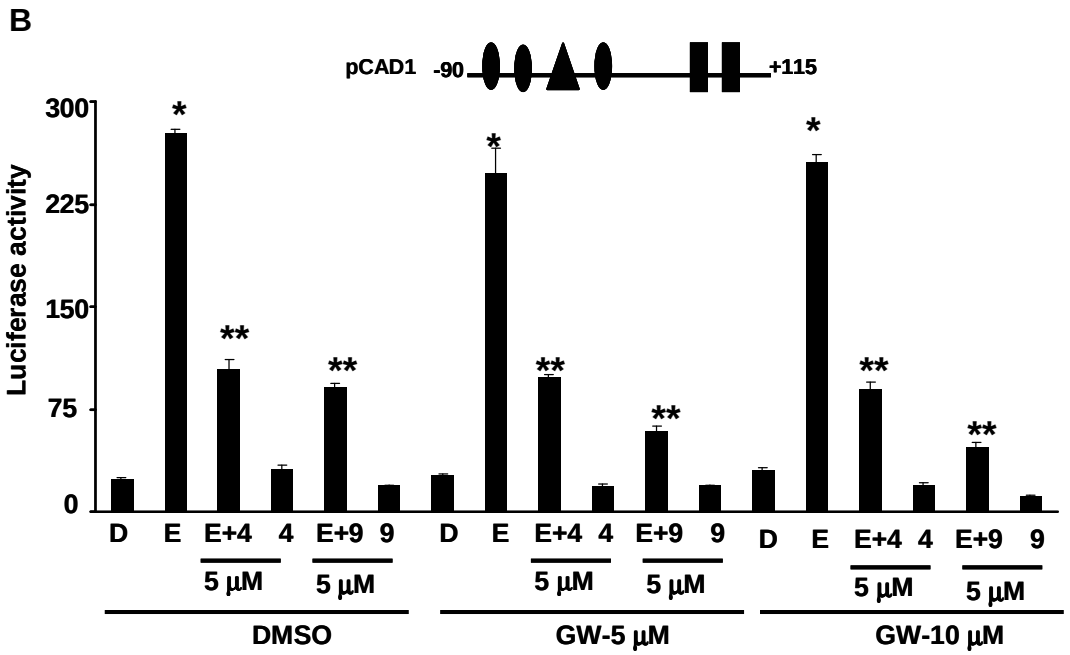


Figure 16. Inhibition of estrogen-induced transactivation in cells transfected with pCAD constructs and treated with DIM-C-pPhtBu (# 4) and DIM-C-pPhC6H5 (# 9) in MCF-7 cells. MCF-7 cells were transfected with pCAD constructs, pCAD1 [A] or pCAD2 [B] treated with DMSO (D), 10 nM E2, # 4 or # 9 alone, or in combination with E2 [E+ #4or E+ # 9] and luciferase activities were determined. Results are expressed as means ± SD for at least three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition by E2 plus # 4 (**) or E2 plus # 9 (**) are indicated.


A
pCAD1
-90
+115
Luciferase activity
300
225
150
75
0
D
E
E+4
4
E+9
9
5 μM
5 μM
DMSO
T007


B
pCAD1
-90
+115
Luciferase activity
300
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D
E
E+4
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E+9
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5 μM
DMSO
GW-5 μM
GW-10 μM

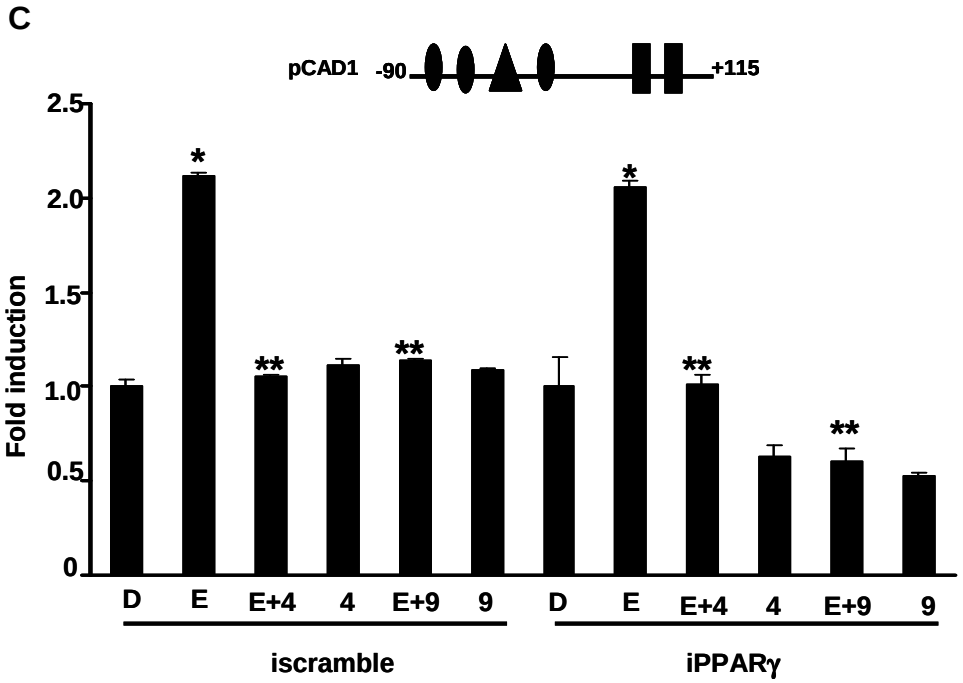

Figure 17. Inhibition of estrogen-induced transactivation in cells transfected with pCAD constructs and treated with DIM-C-pPhtBu (# 4) and DIM-C-pPhC6H5 (# 9) in MCF-7 cells. MCF-7 cells were transfected with pCAD1 construct and treated with DMSO (D), 10 nM E2, E2 plus # 4, E2 plus #9, # 4 or #9 alone, in the presence or absence of PPARγ antagonist T007 [A] or GW9662 [B] and luciferase activities were determined. C. MCF-7 cells were transfected with pCAD1 construct and treated with DMSO (D), 10 nM E2, E2 plus # 4, E2 plus #9, # 4 or #9 alone, in the presence or absence of PPARγ antagonist T007 [A] or GW9662 [B] and luciferase activities were determined. C. RNA interference assay. Cells were transfected with pCAD1and small inhibitory RNA for lamin (nonspecific control) or PPARγ and treated with DMSO (D), 10 nM E2, E2 plus # 4, E2 plus #9, # 4 or #9 alone, and luciferase activity was determined Results are expressed as means ± SD for at least three replicate determinations for each treatment group and significant ($p < 0.05$) induction by E2 (*) or inhibition by E2 plus # 4 (**) or E2 plus # 9 (**) are indicated.

Key Research accomplishments

- Induction of *CAD* gene expression by E2 was significantly downregulated by both TCDD and the pure anti-estrogen ICI in breast cancer cells

- TCDD significantly decreased E2-induced transactivation of GC-rich *CAD* gene promoter constructs in ZR-75 cells. TCDD also inhibited E2-induced luciferase activity of pCAD1m3, containing mutations in a potential DRE, suggesting that this element alone was not responsible for TCDD mediated inhibitory responses .Thus inhibitory AhR/ER crosstalk is functional on the *CAD* gene promoter in MCF-7 and ZR-75 cells and is iDRE-independent

- Proteasome inhibitors such as MG132 alone significantly blocked basal and E2-induced *CAD* mRNA levels and it was not possible to directly determine the effects of ERα restoration on AhR-mediated effects.

- Results of this study demonstrate for the first time that AhR and ERα interact in living MCF-7 and COS-1 cells and this interaction between AhR and ERα is enhanced in presence of E2 plus TCDD. Inhibitory AhR-ERα/Sp1 crosstalk in breast cancer cells cotreated with E2 plus TCDD involves decreased ERα/Sp1 interactions as determined by FRET analysis.

- CHIP data suggested that the loss of ERα from the *CAD* gene promoter may involve recruitment of ERα to AhR-responsive promoters (e.g. CYP1A1) and formation of ERα/AhR complexes. Thus, inhibitory AhR-ERα/Sp1 crosstalk may involve competitive displacement of ERα from the ERα/Sp1 complex by the ligand-activated AhR complex or may result in formation of inactive AhR-ERα/Sp1 complex.

- The PPARγ agonist PGJ2 significantly downregulated E2-induced *CAD* gene mRNA levels and transactivation of *CAD* gene promoter constructs in a PPARγ dependent manner suggesting a possible cross talk between PPARγ and ER signaling pathways.

- PPARγ active, 1,1-Bis(3'indolyl)-1-(p-substitutedphenyl)methanes containing p-t-butyl (DIM-C-pPhtBu), and p-phenyl (DIM-C-pPhC6H5) groups also inhibited E2-mediated transactivation of *CAD* gene promoter constructs, however this inhibition was PPARγ independent.

Reportable Outcomes

(a) Manuscripts, abstracts, presentations

Khan, S., Abdelrahim, M., Samudio, I., and Safe, S., 2003. Estrogen receptor/Sp1 complexes are required for induction of *CAD* gene expression by 17beta-estradiol in breast cancer cells. Endocrinology 144, 2325-2335.

Khan, S., Kim, K., Barhoumi, R., Burghardt, R., Liu, S., and Safe, S. Molecular mechanism of inhibitory aryl hydrocarbon receptor-estrogen receptor/Sp1 crosstalk in breast cancer cells. Mol. Endocrinol. In Press, 2006.

Khan S., Barhoumi, R., Kim, K., Burghardt, R., and Safe, S. Crosstalk: Aryl hydrocarbon receptor and estrogen receptor signaling pathways in breast cancer cells. 96th Annual Meeting of the American Association for Cancer Research, Anaheim, CA. April 16-20, 2005

Khan S., Barhoumi, R., Kim, K., Burghardt, R., and Safe, S. Crosstalk: Aryl hydrocarbon receptor and estrogen receptor signaling pathways in breast cancer cells. Platform competition, College of Veterinary Medicine Research Symposium, Texas A&M University, 2005

Khan, S., Abdelrahim, M., Samudio, I., and Safe, S. Estrogen receptor/Sp1 complexes are required for induction of CAD gene expression by 17beta-estradiol in breast cancer cells. Poster competition, College of Veterinary Medicine Research Symposium, Texas A&M University, 2004

Khan, S., and Safe, S. Mechanism of hormonal regulation of *CAD* gene expression in breast cancer cells. 42nd Annual Meeting of the Society of Toxicology, Salt Lake City, UT, March 9–13, 2003.

(b) Patents/licences applied for or issued
None.

(c) Degrees
Ph.D

(d) Cell lines/serum
No new lines developed.

(e) Informatics
None.

(f) Funding applied

Travel Award, College of Veterinary Medicine, Texas A&M University, 2004

(g) Employment/research opportunities
None.

Conclusions

In summary the results of this research have delineated several important mechanistic aspects of inhibitory AhR-ERα/Sp1 crosstalk. The data demonstrates that inhibitory AhR-ERα/Sp1 crosstalk on the *CAD* gene promoter in breast cancer cells cotreated with E2 plus TCDD involves decreased ERα/Sp1 interactions as determined by FRET analysis. In addition, the loss of ERα from the *CAD* gene promoter is due, in part, to recruitment of ERα to AhR-responsive promoters (e.g. CYP1A1) and formation of ERα/AhR complexes. Thus, inhibitory AhR-ERα/Sp1 crosstalk involves competitive displacement of ERα from the ERα/Sp1 complex by the ligand-activated AhR complex and current studies are investigating the validity of this model for other ERα/Sp1-regulated genes in breast cancer cells. In addition, we are also investigating the molecular mechanisms underlying the antiestrogenic actions of SAhRMs in breast cancer cells.

In this study we also show that PPARγ agonist PGJ2 significantly downregulated E2-induced *CAD* gene mRNA levels and transactivation of *CAD* gene promoter constructs in a PPARγ dependent manner suggesting a possible cross talk between PPARγ and ER signaling pathways. However, DIM-C-pPhtBu and DIM-C-pPhC6H5 inhibited E2-mediated transactivation of *CAD* gene promoter constructs in a PPARγ-independent manner. Current studies are investigating the PPARγ agonists structure-dependent effects on CAD and other key hormone-induced genes in breast cancer cells. Results of this study have increased our understanding of the complex processes underlying the diverse actions of E2 and will facilitate strategies for development of mechanism-based drugs for treatment of breast cancer.

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Appendices

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